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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(54) Title:</b> INSULIN DERIVATIVES<br><br><b>(57) Abstract</b><br><br>Insulin derivatives in which a lipophilic group having from 12 to 40 carbon atoms is attached to the $\alpha$ -amino group of the N-terminal amino acid in the B-chain or to the carboxy group of the C-terminal amino acid in the B-chain have a protracted profile of action.  |  |  |

## INSULIN DERIVATIVES

## FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

## BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the meal-related requirements.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be immunogenic in man and that their use for medical purposes may

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe<sup>B1</sup> or to the  $\epsilon$ -amino group of Lys<sup>B29</sup> or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

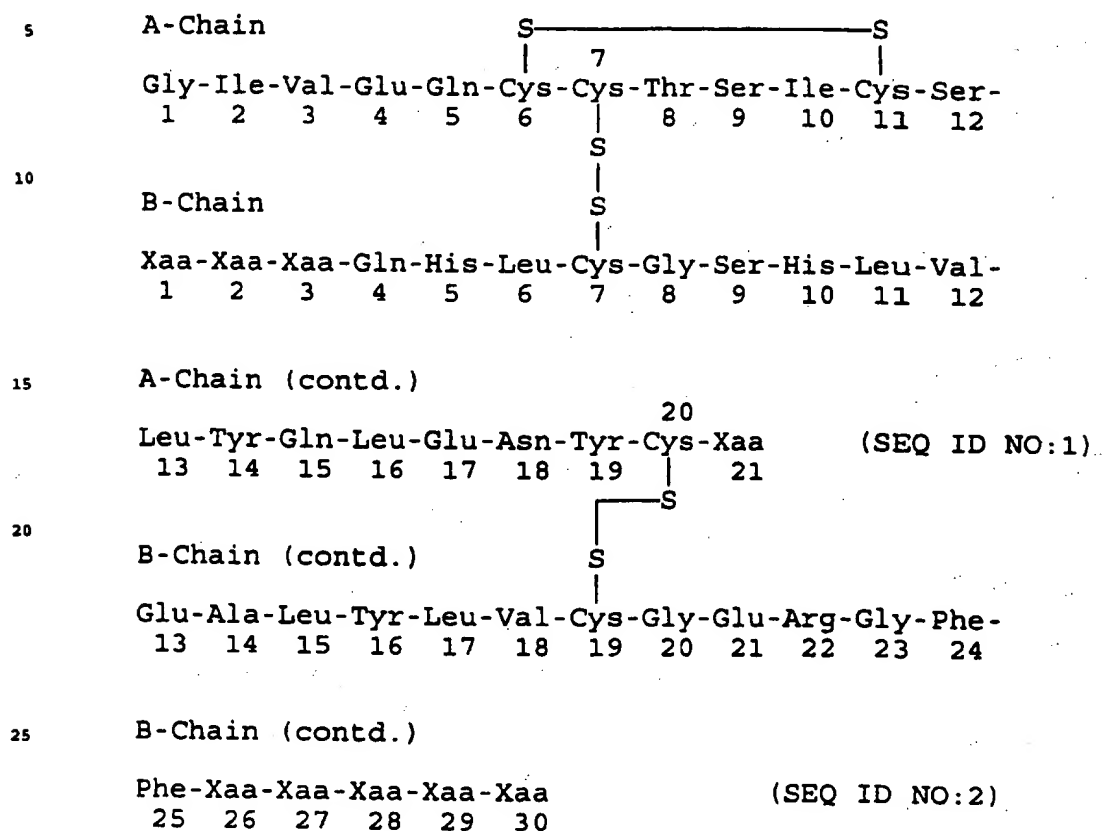
Insulins, which in the B30 position has an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin  
10 analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

US 5,359,030 (Ekwuribe, Protein Delivery, Inc.) describes conjugation-stabilized polypeptide compositions for oral or  
15 parenteral administration comprising a polypeptide covalently coupled with a polymer including a linear polyalkylene moiety and a lipophilic moiety, said moieties being arranged so relative to each other that the polypeptide has an enhanced in vivo resistance to enzymatic degradation.

20 EP 511600 A2 relates i.a. to protein derivatives of the formula [protein][Z]<sub>n</sub>, wherein [protein] represents a protein having n amino residues each derivable from an amino group by removal of one of its hydrogen atoms, in stead of amino groups, [Z] is a residue represented by the formula -CO-W-COOH wherein W is a  
25 divalent long chain hydrocarbon group which may also contain certain hetero atoms and n represents an average of the number of amide bonds between [Z] and [protein]. It is mentioned that the protein derivatives of the invention have an extremely prolonged serum half-life as compared with the proteins from  
30 which they are derived and that they exhibit no antigenicity. It is also mentioned, that insulin is one of the proteins from which derivatives according to the invention can be made, but no specific insulin derivatives are disclosed in EP 511600 nor is there any indication of a preferred [Z] or (a) preferred

carbon atoms attached, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:



wherein

Xaa at position A21 is any codable amino acid except  
30 Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28 and B29 are, independent of each other, any codable amino acid except Cys or deleted;

Xaa at position B30 is any codable amino acid except Cys, a dipeptide comprising no Cys or Arg, a tripeptide comprising no Cys or Arg, a tetrapeptide comprising no Cys or Arg or deleted; and either the amino group of the N-terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally

A-Chain (contd.)

Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Xaa (SEQ ID NO:1)  
 13 14 15 16 17 18 19 20 21

5

B-Chain (contd.)

Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-  
 13 14 15 16 17 18 19 20 21 22 23 24

10

B-Chain (contd.)

Phe-Xaa-Xaa-Xaa-Xaa-Xaa (SEQ ID NO:2)  
 25 26 27 28 29 30

wherein

15 Xaa at position A21 is any codable amino acid except Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28, B29 and B30 are, independent of each other, any codable amino acid except Cys or deleted; and either the amino group of the N-  
 20 terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged or the carboxyl group of the C-terminal amino acid of the B-chain has a lipophilic group, Z, attached to it which group has from  
 25 12 to 40 carbon atoms and optionally contains a group which can be negatively charged with the proviso that if one or more of the amino acids at position B1, B2 and B3 is (are) deleted then the number of the N-terminal amino acid is found by counting down from Cys<sup>B7</sup> which is always assigned the number 7 and that

30 (a) when B1-B2-B3 is Phe-Val-Asn and A21 is Asn and B26-B27-B28-B29-B30 is Tyr-Thr-Pro-Lys-Thr or Tyr-Thr-Pro-Lys-Ala, then said W or Z always contains a group which can be negatively charged; and

(b) when B29 and B30 are deleted and a group Z as defined above  
 35 is present at the C-terminal amino acid of the B-chain and neither B1, B2 nor B3 is deleted then B1-B2 is different from

selected from the group comprising Ala, Asn, Gln, Glu, Gly and Ser.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B1 is Phe.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B1 is deleted.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B2 is selected from the group comprising Ala and Val.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B3 is selected from the group comprising Asn, Gln, Glu and Thr.

15 In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B26 is Tyr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B27 is Thr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Pro.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B29 is Lys or Thr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Lys and the amino acid at position B29 is Pro.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is des(B30) human insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B28-B30) insulin.

10 In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B27-B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B26-B30)  
15 insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Pro and the amino acid at position B29 is Thr.

In another preferred embodiment, the invention relates to an  
20 insulin derivative which has a group, W, as mentioned above, attached to the N-terminal  $\alpha$ -amino group of its B-chain, W being a group of the general formula  $\text{CH}_3(\text{CH}_2)_n\text{CH}(\text{COOH})\text{NH}-\text{CO}(\text{CH}_2)_r\text{CO}-$  wherein n is an integer from 9 to 15.

In another preferred embodiment, the invention relates to an  
25 insulin derivative which has a group, W, as mentioned above, attached to the N-terminal  $\alpha$ -amino group of its B-chain, W being a group of the general formula  $\text{CH}_3(\text{CH}_2)_r\text{CO}-\text{NHCH}(\text{COOH})(\text{CH}_2)_n\text{CO}-$  wherein r is an integer from 9 to 15.

In another preferred embodiment, the invention relates to an  
30 insulin derivative which has a group, W, as mentioned above,



In another preferred embodiment, the invention relates to des(B28-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to des(B27-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to des(B26-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

10 In another preferred embodiment, the invention relates to the use of an insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a  
15 patient in need of such a treatment comprising a therapeutically effective amount of an insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a  
20 pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of an insulin derivative according to the invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together  
25 with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising an insulin derivative according to the invention which is soluble at physiological pH values.

30 In another preferred embodiment, the invention relates to a pharmaceutical composition comprising an insulin derivative

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawing wherein

Fig. 1 shows the construction of the plasmids pKV153, pKV159, pJB173, pJB174 and pJB175;

Fig. 2a which is continued in Fig. 2b shows the sequence of pMT742, position 907 to 1500, and the oligonucleotides #94, #593, #2371 and #3075 used for PCR1A, PCR1B and PCR1C of Example 1. The 138 amino acid sequence corresponding to the MF  
10 alpha prepro-leader (amino acids Nos. 1-85) and an insulin precursor which has the amino acid sequence B(1-29)AlaAlaLysA(1-21) wherein A(1-21) is the A chain of human insulin and B(1-29) is the B chain of human insulin in which Thr(B30) is missing, is shown below the coding sequence (amino  
15 acids Nos. 86-138).

## DETAILED DESCRIPTION OF THE INVENTION

### Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243, p.  
20 3558 (1968).

In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used:

DMSO for dimethyl sulphoxide,  
25 DMF for dimethylformamide,  
Boc for tert-butoxycarbonyl,  
NMP for 1-methyl-2-pyrrolidone,  
TFA for trifluoroacetic acid,  
X-OSu for an N-hydroxysuccinimid ester,  
30 X for an acyl group,

(A1,B29)-diBoc des(B30) insulin. After an optional purification, e.g. by HPLC, an acyl group is introduced in the  $\alpha$ -amino group of the amino acid in position B1 by allowing the product to react with a N-hydroxysuccinimide ester of the formula W-OSu wherein W is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, (N<sup>B1</sup>-W) des(B30) insulin, is isolated.

## 2.2 Starting from a single chain human insulin precursor.

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and which has a bridge from a C-terminal lysine in position B26, B27, B28 or B30 to A1 can be used as starting material. Preferably, the bridge is a peptide of the formula Y<sub>n</sub>-Arg, where Y is a codable amino acid except cysteine, lysine and arginine, and n is zero or an integer between 1 and 35. When n>1, the Y's may designate different amino acids. Preferred examples of the bridge from Lys in position B26, B27, B28 or B30 to A1 are: AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No. 163529).

Treatment of such a precursor of the general formula Ext-Arg-B(1-Q)-Y<sub>n</sub>-Arg-A(1-21), wherein Q is 26, 27, 28 or 30, with a lysyl endopeptidase, e.g. *Achromobacter lyticus* protease, yields Ext-Arg-B(1-Q) Y<sub>n</sub>-Arg-A(1-21) insulin. Acylation of this intermediate with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the  $\epsilon$ -amino group of Lys<sup>B0</sup>, and in the N-terminal amino group of the A-chain and the B-chain to give (N<sup>B0</sup>-X) X-Ext-Arg-B(1-Q) X-Y<sub>n</sub>-Arg-A(1-21) insulin. This intermediate on treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, Z-human insulin wherein Z is Lys<sup>B0</sup>-X.

Examples of suitable buffers are sodium acetate and sodium phosphate.

Preferred pharmaceutical compositions of the particular insulins of the present invention are solutions hexameric complexes. Typically the hexameric complexes are stabilized by two or more zinc ions and three or more molecules of a phenolic compound like phenol or meta-cresol or mixtures thereof per hexamer.

In a particular embodiment, a composition is provided which contains two different insulins, one having a protracted profile of action and one having a rapid onset of action, in the form of soluble hexameric complexes. Typically the hexameric complexes are stabilized by two or more zinc ions and three or more molecules of a phenolic compound like phenol or meta-cresol or mixtures thereof per hexamer. The complexes are mixtures of hexamers of the particular insulins and mixed hexamers in which the ratio between the two different insulins is from 1:5 to 5:1.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Synthetic DNA fragments were synthesised on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron Letters 22 (1981) 1859-1869).

All other methods and materials used common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York, 1989).

#### 10 Analytical

Molecular masses of insulin precursors prepared were obtained by mass spectroscopy (MS), either by plasma desorption mass spectrometry (PDMS) using Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or electrospray mass spectrometry (ESMS) using an API III Biomolecular Mass Analyzer (Perkin Elmer Sciex Instruments, Thornhill, Canada).

The lipophilicity of an insulin derivative relative to human insulin,  $k'_{rel}$ , was measured on a LiChrosorb® RP18 (5 $\mu$ m, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water. The elution was monitored by the absorption of the eluate at 214 nm. Void time,  $t_0$ , was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin,  $t_{human}$ , was adjusted to at least  $2t_0$  by varying the ratio between the A and B solutions.  $k'_{rel}$  is defined as  $(t_{derivative} - t_0) / (t_{human} - t_0)$ .

As a measure of the protraction of the compounds of the invention, the disappearance rate in pigs was studied and  $T_{50\%}$  was determined.  $T_{50\%}$  is the time when 50% of the A14 Tyr(<sup>125</sup>I)-labeled analogue has disappeared from the site of injection as measured with an external  $\gamma$ -counter (Ribet, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P.J. Lefebvre (Eds): Diabetes 1985; Proceedings of the

10.0  $\mu$ l of 10x PCR buffer  
10.0  $\mu$ l of 2.5 mM dNTP  
0.5  $\mu$ l of Taq polymerase enzyme  
71.3  $\mu$ l of water

5 PCR1B:

0.2  $\mu$ l of pMT742 plasmid template  
4.0  $\mu$ l of oligonucleotide #3075 (100 pmol)  
4.0  $\mu$ l of oligonucleotide #2371 (100 pmol)  
10.0  $\mu$ l of 10x PCR buffer  
10 10.0  $\mu$ l of 2.5 mM dNTP  
0.5  $\mu$ l of Taq polymerase enzyme  
71.3  $\mu$ l of water

In both cases two cycles were performed at 94 °C for 1 min., 45 °C for 1 min. and 72 °C for 1 min. and subsequently followed by  
15 11 cycles: 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1 min.

20  $\mu$ l of each PCR mixture was loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour  
20 Laboratory Press, 1989). Resulting DNA fragments (452 bp from PCR1A and 170 bp from PCR1B) were cut out of the agarose gel and isolated using the Gene Clean kit (Bio101 Inc., PO BOX 2284, La Jolla, CA, USA) according to the manufacturer's instructions. The purified DNA fragments were dissolved in 100  
25  $\mu$ l of water.

The following PCR was performed

PCR1C:

1.0  $\mu$ l of DNA fragment from PCR1A  
30 1.0  $\mu$ l of DNA fragment from PCR1B  
10.0  $\mu$ l of 10 x PCR buffer  
10.0  $\mu$ l of 2.5 mM dNTP

XbaI). The selected plasmid designated pKV153 was shown by DNA sequencing analysis (using the Sequenase kit from U.S. Biochemical Corp. according to the manufacturer's recommendations) to contain the correct sequence encoding the MFalphaprepro-leader/Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

pKV153 was transformed into *S. cerevisiae* strain MT663 and selected for growth on glucose as described in European patent application having the publication No. 214826.

The resulting yeast strain was designated yKV153 and was verified to produce Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor in the culture media by HPLC and mass spectroscopy.

## 15 EXAMPLE 2

Synthesis of Lys<sup>B30</sup>(N'-tetradecanoyl) Thr<sup>B29</sup> human insulin.

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2a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV159 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#3881 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC  
ACTCCTACCAAGTCTGACGATGCTAGAGGTATTGTCG-3'

25 #2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The following Polymerase Chain Reaction (PCR) was performed using Gene Amp PCR reagent kit as described above.

PCR2:

The selected plasmid designated pKV159 was shown by DNA sequencing analysis to contain the correct sequence encoding the MF alpha prepro-leader/Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

pKV159 was transformed into *S. cerevisiae* strain MT663 and selected for growth on glucose as described.

The resulting yeast strain was designated yKV159 and was verified to produce Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor in the culture media by HPLC and mass spectroscopy.

2b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

15 The yKV159 strain was fermented for 72 hours and five litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the insulin precursor was concentrated on a Pharmacia Streamline® 50 column packed with 300 ml of  
20 Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH value 3.6, the precursor was eluted by 0.5 M NH<sub>4</sub> and the fraction from 300 to 600 ml was collected. The pH value was adjusted to 2.5 and the precursor was purified by RP-HPLC using 15 µ spherical C18 silica of 100 Å pore size and 0.2 M Na<sub>2</sub>SO<sub>4</sub>,  
25 0.04 M H<sub>3</sub>PO<sub>4</sub> as buffer, and using a gradient from 23 to 33% acetonitrile. The precursor eluted at about 27-28% acetonitrile. The pool containing the central major peak of the precursor was desalted by gel filtration on Sephadex® G-50 F in 0.5 M acetic acid, and the precursor isolated by  
30 lyophilization. Yield: 486 mg.

2c. Synthesis of Ala-Thr-Arg-B(1-28)-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.



filtration on Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 91 mg.

Molecular mass of the title compound, found by MS:  $6020 \pm 6$ , theory: 6018.

5 Molecular mass of the B-chain, found by MS:  $3642 \pm 5$ , theory: 3640.

Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS:  $1326 \pm 2$ , theory: 1326.

Relative lipophilicity,  $k'_{rel} = 113$ .

10 Disappearance half-life,  $T_{50\%}$ , after subcutaneous injection in pigs:  $20.3 \pm 5.2$  hours ( $n=6$ ).

### EXAMPLE 3

Synthesis of Lys<sup>B28</sup> (N'-tetradecanoyl) des(B29-B30) human insulin.

---

15 3a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yJB173 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

20 #627 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGC GGTGAAAGAGGTTTCTTC  
TACACTAAGTCTGACGATGCTAG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed  
25 in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #627.

The resulting plasmid was designated pJB173 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-

M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 110 mg.

3d. Synthesis of  $N^{A-5}, N^{B-3}, N^{B28}$ -tris(tetradecanoyl) Ala-Thr-Arg-B(1-27)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

5 110 mg of the double-chain, extended insulin obtained as described above were dissolved in a mixture of 0.84 ml of DMSO and 0.275 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.185 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added.  
10 After 2 hour at 15°C, the reaction was stopped by addition of 32.5 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

3e. Synthesis of Lys<sup>B28</sup> (N'-tetradecanoyl) des(B29-B30) human  
15 insulin.

To the resulting solution from the previous step was added 1.5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 18 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the solution  
20 applied to a 1.5 x 21 cm column of QAE-Sephadex<sup>®</sup> A-25. Isocratic elution was performed at a rate of 10 ml/h using a 0.12 M  $NH_4Cl$  buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with  $NH_3$ . The title compound emerged from the column after 250-390 ml, peaking at 330 ml. Finally, the buffer was changed to 0.01  
25 M  $NH_4HCO_3$  by gel filtration using Sephadex<sup>®</sup> G-50 Fine, and the product was isolated in the dry state by lyophilization. Yield: 47 mg.

Molecular mass of the title compound, found by MS: 5820  $\pm$  2, theory: 5819.

30 Molecular mass of the B-chain, found by MS: 3444  $\pm$  4, theory: 3442.

Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1128  $\pm$  2, theory: 1128.

centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the solution was diluted with water to 8 litres in order to decrease the salt concentration. The resulting conductivity was 7.9 mS/cm. The insulin precursor was concentrated using a Pharmacia Streamline® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH 3.6, the precursor was eluted by 0.5 M NH<sub>3</sub> and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated in vacuo at room temperature and the pH value of the resulting 280 ml was adjusted to 9.0 with hydrochloric acid.

4c. Synthesis of Ala-Thr-Arg-B(1-26)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 280 ml of solution of the single-chain precursor obtained as described above was added 3 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 13 hours at 30°C the gel was removed by filtration. The pH value was adjusted to 2.5 and the solution was filtered through a Milipore® 0.45 µ filter. The double-chain, extended insulin was purified in 4 runs by RP-HPLC using a 2x20 cm column packed with 15 µ spherical C18 silica of 100 Å pore size and 0.2 M Na<sub>2</sub>SO<sub>4</sub>, 0.04 M H<sub>3</sub>PO<sub>4</sub>, pH 2.5 as buffer, and using a gradient from 24 to 33% acetonitrile. The double-chain, extended insulin eluted at about 30-31% acetonitrile. The acetonitrile was removed from the combined pools by evaporation in vacuo, and the salts were removed by gelfiltration using a 5x47 cm column of Sephadex G-25 in 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 69 mg.

4d. Synthesis of N<sup>α</sup>-5, N<sup>α</sup>B-3, N<sup>ε</sup>B27-tris(tetradecanoyl) Ala-Thr-Arg-B(1-26)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

62 mg of the double-chain, extended insulin obtained as described under 4e was dissolved in a mixture of 0.44 ml of DMSO and 0.15 ml of 2 M diisopropylethylamine in NMP. The

5a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yJB175 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#629 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTC  
AAAGTCTGACGATGCTAG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #629.

The resulting plasmid was designated pJB175 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yJB175.

5b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yJB175 strain was fermented for 72 hours and 3.7 litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the solution was diluted with water to 8.5 litres in order to decrease the salt concentration. The resulting conductivity was 7.7 mS/cm. The insulin precursor was concentrated using a Pharmacia Strealine® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH 3.6, the precursor was eluted by 0.5 M ammonia and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated in vacuo at room temperature and the pH

To the solution from the previous step was added 1 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 23 hours the gel was removed by filtration, the pH value was adjusted to 9.0 and the solution applied to a 1.5 x 25.5 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 19.3 ml/h using a 0.12 M NH<sub>4</sub>Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with ammonia. The title compound emerged from the column after 320 ml, and a fraction from 320 to 535 ml was collected. Finally, the buffer was changed to 0.01 M NH<sub>4</sub>HCO<sub>3</sub> by gel filtration on Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 25 mg.

Molecular mass of the title compound found by MS: 5555 ± 6, theory: 5555.

Molecular mass of the B-chain found by MS: 3179 ± 4, theory: 3178.

Molecular mass of the C-terminal fragment of B-chain digested by V8 protease found by MS: 864 ± 1, theory: 863.5.

Relative lipophilicity,  $k'_{rel}$  = 151.

Disappearance half-life,  $T_{50\%}$ , after subcutaneous injection in pigs: 14.4 ± 1.5 h (n = 5).

#### EXAMPLE 6

Synthesis of (N(1-carboxytridecyl)-2-amidosuccinyl)-Phe<sup>98</sup>: des(B30) human insulin.

25

A1,B29-diBoc-des(B30) human insulin (200 mg, 0.033 mmol) was dissolved in DMF (15 ml) and triethylamine (20 µl) was added. N(1-carbomethoxytridecyl)-2-amidosuccinic acid N-hydroxysuccinimide ester (16 mg, 0.033 mmol) was added and after 4 hours at room temperature the reaction mixture was evaporated in vacuo to dryness. The Boc groups were removed by treatment for 30 min at room temperature with trifluoroacetic acid (5 ml). The trifluoroacetic acid was removed by evaporation in vacuo. The residue was dissolved at 0°C in 0.1

temperature with trifluoroacetic acid (5 ml). The trifluoroacetic acid was removed by evaporation in vacuo. The title compound was purified from the precipitate by RP-HPLC using a C18 silica column and eluting with a linear gradient from 16 to 64% acetonitrile in a 50 mM Tris/phosphate buffer containing 75 mM  $(\text{NH}_4)_2\text{SO}_4$  at pH 7. The title compound emerged from the column at about 50% acetonitrile. The acetonitrile was evaporated in vacuo, and ethanol was added to 20% (v/v). Adjustment of the pH value to 5.0 caused the product to precipitate. After centrifugation the precipitate was dissolved in 10 mM  $\text{NH}_4\text{HCO}_3$ , desalted by gel filtration using Sephadex G-25 and lyophilized. Yield: 97 mg. Molecular mass, found by PDMS: 6105, theory: 6104.

#### EXAMPLE 8

15 Synthesis of Gly<sup>B28</sup>, Thr<sup>B29</sup>, Lys<sup>B30</sup> (N'-tetradecanoyl) human insulin.

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8a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV195 using the *S. cerevisiae* MF  
20 alpha prepro-leader.

The following oligonucleotides were synthesised:

#4790 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC  
ACTGGTACCAAGTCTGACGATGCTAGAGGTATTGTCG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

25 The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #4790.

salt were removed by gelfiltration using a 5x47 cm column of Sephadex® G-25 and 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 176 mg.

8d. Synthesis of N<sup>A-5</sup>, N<sup>B-3</sup>, N<sup>B30</sup>-tris(tetradecanoyl) Ala-Thr-Arg-  
s B(1-27)-Gly-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

176 mg of the double-chain, extended insulin was dissolved in a mixture of 1.4 ml of DMSO and 0.275 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.963 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide  
10 ester in DMSO/NMP (1:1, v/v) was added. After 20 hour at 15°C the reaction was stopped by addition of 50 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

8e. Synthesis of Gly<sup>B28</sup>, Thr<sup>B29</sup>, Lys<sup>B30</sup> (N<sup>t</sup>-tetradecanoyl) human  
15 insulin.

To the solution from the previous step was added 2.5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 5 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the solution  
20 applied to a 1.5 x 26.5 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 9.3 ml/h using a 0.12 M NH<sub>4</sub>Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with ammonia. The title compound emerged from the column after 325-455 ml, peaking at 380 ml. Finally, the buffer  
25 was changed to 0.01 M NH<sub>4</sub>HCO<sub>3</sub> by gel filtration using Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 50 mg.

Molecular mass of the title compound, found by MS: 5979 ± 6, theory: 5977.

30 Molecular mass of the B-chain, found by MS: 3600 ± 4, theory: 3600.

Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1286 ± 2, theory: 1286.

sulphuric acid and 3.4 litres of water were added to dilute salts to a conductivity of 7.7 mS/cm. The insulin precursor was concentrated to 300 ml using the procedure described in Example 8b.

9c. Synthesis of Ala-Thr-Arg-B(1-27)-Gly-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 300 ml solution at pH 9.0 containing 390 mg of the single-chain precursor were added 5 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 40 hours at 30°C, the gel was removed by filtration. The pH value was adjusted to 3.5 and the solution was filtered through a Milipore® 0.45 µ filter. The double-chain, extended insulin was purified in 3 runs by RP-HPLC using a 2x20 cm column packed with 10 µ spherical C18 silica of 120 Å pore size and 0.2 M Na<sub>2</sub>SO<sub>4</sub>, 0.04 M H<sub>3</sub>PO<sub>4</sub>, pH 3.5 as buffer, and using a gradient from 23 to 33% acetonitrile at a rate of 4 ml/min and a column temperature of 40°C. The double-chain, extended insulin eluted at about 29% acetonitrile. The acetonitrile was removed from the combined pools of 60 ml by evaporation in vacuo, and the salt were removed by gelfiltration using a 5x47 cm column of Sephadex® G-25 and 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 154 mg.

9d. Synthesis of N<sup>αA-5</sup>, N<sup>αB-3</sup>, N<sup>αB29</sup>-tris(tetradecanoyl) Ala-Thr-Arg-B(1-27)-Gly-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

154 mg of the double-chain, extended insulin was dissolved in a mixture of 1.05 ml of DMSO and 0.329 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.22 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hour at 15°C, the reaction was stopped by addition of 40 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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10 (I) TELEX: 37173

(ii) TITLE OF INVENTION: ACYLATED INSULIN

(iii) NUMBER OF SEQUENCES: 2

## (iv) CORRESPONDENCE ADDRESS:

- 15 (A) ADDRESSEE: Novo Nordisk A/S  
Corporate Patents  
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(E) COUNTRY: Denmark

## (v) COMPUTER READABLE FORM:

- 20 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

- 25 (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

- 30 (A) APPLICATION NUMBERS: DK 0276/95  
(B) FILING DATES: 17-MAR-1995

## (viii) ATTORNEY/AGENT INFORMATION:

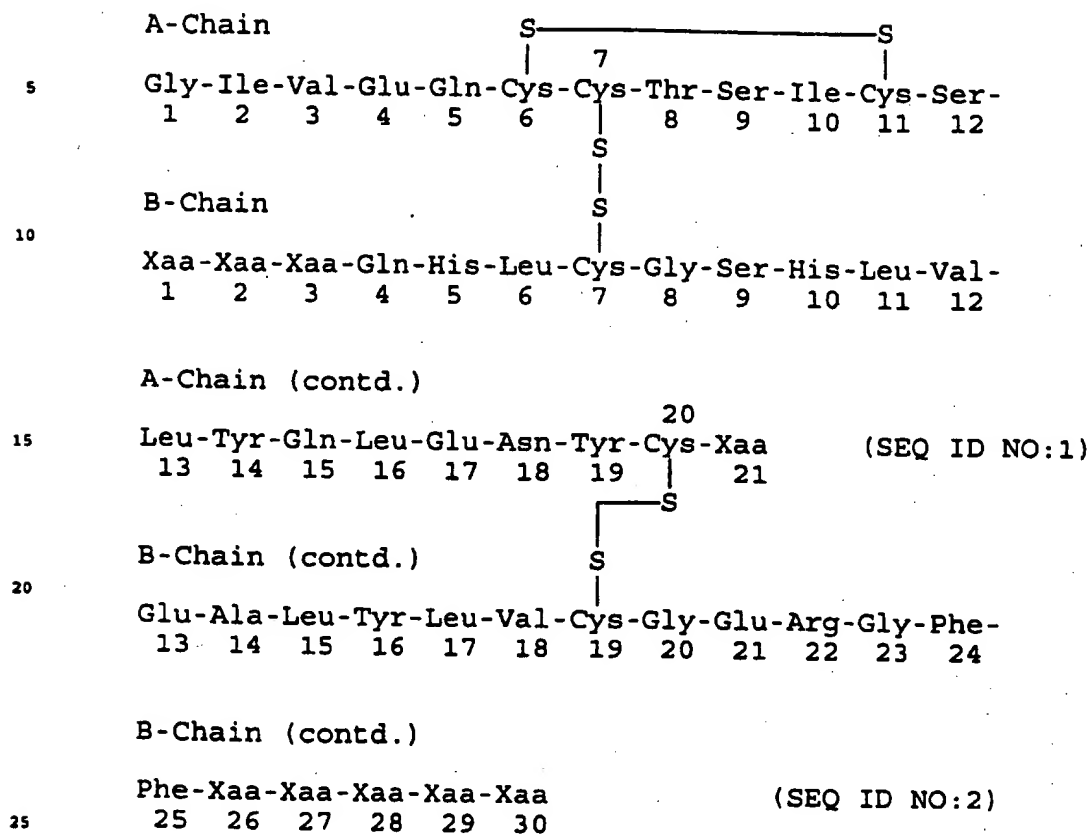
- (A) NAME: Jørgensen, Dan et al.  
(C) REFERENCE/DOCKET NUMBER: 4341-WO,DJ

## (ix) TELECOMMUNICATION INFORMATION:

- 35 (A) TELEPHONE: +45 44448888  
(B) TELEFAX: +45 44493256

## CLAIMS

1. An insulin derivative having the following sequence:



wherein

Xaa at position A21 is any codable amino acid except Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28 and B29 are,  
 30 independent of each other, any codable amino acid except Cys or deleted;

Xaa at position B30 is any codable amino acid except Cys, a dipeptide comprising no Cys or Arg, a tripeptide comprising no Cys or Arg, a tetrapeptide comprising no Cys or Arg or  
 35 deleted; and either the amino group of the N-terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged or the carboxyl group of the C-terminal amino acid of the B-chain has

5. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B1 designates Phe or is deleted.
6. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B2 designates Ala or Val.
7. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B3 designates an amino acid selected from the group comprising Asn, Gln, Glu, and Thr.
8. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B26 designates Tyr.
9. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B27 designates Thr.
10. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Pro.
11. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B29 designates Lys or Thr.
12. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B30 designates Thr or  $\epsilon$ -acylated Lys.
13. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B30 is deleted.
14. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Lys and Xaa at position B29 designates Pro.
15. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Pro and Xaa at position B29 designates Thr.

26. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a therapeutically effective amount of an insulin derivative according to claim 1, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

27. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

28. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

2 / 3

#94 !

5' -TAAATCTATAACTACAAAAACACATA-3' EcoRI

5' -CTTAAATCTATAACTACAAAAACACATACAGGAATTCATTCAAGAATAGTTCAAACAA

907 -----+-----+-----+-----+-----+-----+-----+-----+----- 966

3' -GAATTTAGATATTGATGTTTTTTGTGTATGTCCTTAAGGTAAGTTCTTATCAAGTTTGTT

GAAGATTACAACTATCAATTTTCATACACAATATAAACGATTAAAAGAATGAGATTTTCCT

967 -----+-----+-----+-----+-----+-----+-----+-----+----- 1026

CTTCTAATGTTTGATAGTTAAAGTATGTGTTATATTGCTAATTTTCTTACTCTAAAGGA

MetArgPhePro 4

TCTATTTTTACTGCTGTTTTATTTCGCTGCTTCCTCCGCTTTAGCTGCTCCAGTCAACACT

1027 -----+-----+-----+-----+-----+-----+-----+-----+----- 1086

AGATAAAATGACGACAAAATAAGCGACGAAGGAGGCGAAATCGACGAGGTCAGTTGTGA

SerIlePheThrAlaValLeuPheAlaAlaSerSerAlaLeuAlaAlaProValAsnThr 24

ACCACTGAAGATGAAACGGCTCAAATTCAGCTGAAGCTGTCATCGGTTACTCTGATTTA

1087 -----+-----+-----+-----+-----+-----+-----+-----+----- 1146

TGGTGACTTCTACTTTGCCGAGTTTAAGGTCGACTTCGACAGTAGCCAATGAGACTAAAT

ThrThrGluAspGluThrAlaGlnIleProAlaGluAlaValIleGlyTyrSerAspLeu 44

GAAGGTGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACCTCCACCAATAACGGTTTATTG

1147 -----+-----+-----+-----+-----+-----+-----+-----+----- 1206

CTTCCACTAAAGCTACAACGACAAAACGGTAAAGGTTGAGGTGGTTATTGCCAAATAAC

GluGlyAspPheAspValAlaValLeuProPheSerAsnSerThrAsnAsnGlyLeuLeu 64

TTTATCAATACTACTATTGCCTCCATTGCTGCTAAAGAAGAAGGTGTTTCTTTGGATAAA

1207 -----+-----+-----+-----+-----+-----+-----+-----+----- 1266

AAATAGTTATGATGATAACGGAGGTAACGACGATTTCTTCTCCACAAAGAAACCTATTT

PheIleAsnThrThrIleAlaSerIleAlaAlaLysGluGluGlyValSerLeuAspLys 84

3' -CCACAAAGAAACCTATTT

HindIII

5' -TTGGTTGAAGCTTTGTACTTGTTTGC

AGATTTCGTTAACCAACACTTGTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGTTTGC

1267 -----+-----+-----+-----+-----+-----+-----+-----+----- 1326

TCTAAGCAATTGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACCAAACG

ArgPheValAsnGlnHisLeuCysGlySerHisLeuValGluAlaLeuTyrLeuValCys 104

TCT GCAATTGGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACC-5'

C A #593 !

T ATGTAGCCTTTGGT

T T TGACGATGCT

CTTCGACTTCGACTTCGAC C A

#3075 ! T G

GGTGAAAGAGGTTTCTTCTACACTCCTAAG AGGTATTG-3'

GGTGAAAGAGGTTTCTTCTACACTCCTAAGGCTGCTAAGGGTATTGTGCGAACAATGCTGT

1327 -----+-----+-----+-----+-----+-----+-----+-----+----- 1386

CCACTTTCTCCAAAGAAGATGTGAGGATTCCGACGATTCCCATAACAGCTTGTTACGACA

GlyGluArgGlyPhePheTyrThrProLysAlaAlaLysGlyIleValGluGlnCysCys 124

ACCTCCATCTGCTCCTTGTACCAATTGGAAACTACTGCAACTAGACGCAGCCCGCAGGC

1387 -----+-----+-----+-----+-----+-----+-----+-----+----- 1446

TGGAGGTAGACGAGGAACATGGTTAACCTTTTGATGACGTTGATCTGCGTCGGGCGTCCG

ThrSerIleCysSerLeuTyrGlnLeuGluAsnTyrCysAsn\*\*\* 3' -GGGCGTCCG 138

Fig. 2a

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00107

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, A61K 28/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CAPLUS, MEDLINE, WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| P,X       | WO 9507931 A1 (NOVO NORDISK A/S), 23 March 1995<br>(23.03.95)   | 1-11                  |
|           | --  |                       |
| X         | Patent Abstracts of Japan, Vol 14, No 7, C-673,<br>abstract of JP, A, 1-254699 (KODAMA K.K.),<br>11 October 1989 (11.10.89) | 1-11                  |
|           | --  |                       |
| A         | WO 9200321 A1 (NOVO NORDISK A/S), 9 January 1992<br>(09.01.92)  | 1-24                  |
|           | --  |                       |
| A         | EP 0376156 A2 (HOECHST AKTIENGESELLSCHAFT),<br>4 July 1990 (04.07.90)   | 1-24                  |
|           | --  |                       |
|           | -----   |                       |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
- \* "E" earlier document but published on or after the international filing date
- \* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \* "O" document referring to an oral disclosure, use, exhibition or other means
- \* "P" document published prior to the international filing date but later than the priority date claimed

\* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\* "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\* "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\* "&" document member of the same patent family

Date of the actual completion of the international search

12 July 1996

Name and mailing address of the ISA/  
Swedish Patent Office  
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Facsimile No. +46 8 666 02 86

Date of mailing of the international search report

15-07-1996

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.

PCT/DK 96/00107

| Patent document<br>cited in search report |         | Publication<br>date | Patent family<br>member(s) |          | Publication<br>date |
|---|---------|---------------------|----------------------------|----------|---------------------|
| WO-A1-                                    | 9507931 | 23/03/95            | NONE                       |          |                     |
| WO-A1-                                    | 9200321 | 09/01/92            | AU-A-                      | 8054391  | 23/01/92            |
|   |         |                     | EP-A,A-                    | 0536245  | 14/04/93            |
|   |         |                     | JP-T-                      | 5508406  | 25/11/93            |
| EP-A2-                                    | 0376156 | 04/07/90            | AT-T-                      | 136038   | 15/04/96            |
|   |         |                     | AU-B,B-                    | 623963   | 28/05/92            |
|   |         |                     | AU-A-                      | 4733289  | 05/07/90            |
|   |         |                     | CA-A-                      | 2006818  | 29/06/90            |
|   |         |                     | DE-A-                      | 3844211  | 05/07/90            |
|   |         |                     | DE-D-                      | 58909633 | 00/00/00            |
|   |         |                     | IL-A-                      | 92905    | 21/10/94            |
|   |         |                     | JP-A-                      | 2225498  | 07/09/90            |
|   |         |                     | PT-B-                      | 92757    | 29/12/95            |
|   |         |                     | US-A-                      | 5506202  | 09/04/96            |